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Estrogenic Responses to Xenobiotics in Channel Catfish (*Ictalurus punctatus*)

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ABSTRACT

Several environmentally relevant contaminants are characterized as xenoestrogens by virtue of their ability to induce responses similar to 17ß-estradiol (E2). There is concern that exposure to these xenoestrogens may result in endocrine and, thus, reproductive disruption. The objective of the present research was to determine whether xenobiotics known to elicit estrogenic responses in mammals were estrogenic in channel catfish, and if these compounds were capable of altering normal physiological response to E2. Sexually immature catfish were exposed by intraperitoneal injection to E2, suspected xenoestrogens or combination doses of E2 and xenoestrogen. Appearance of vitellogenin (Vg) in serum was used as the bioindicator of estrogenicity; Vg was measured by ELISA 7 days after injection. The ED_{50} of E2 for the appearance of Vg in blood, 0.6 mg/kg, was used as the positive control. Synthetic estrogens, ethinylestradiol, mestranol and DES were effective in inducing Vg; the antiestrogen tamoxifen inhibited response to E2 when given in a combination dose. Treatment by both methoxychlor and p-*nonylphenol resulted in Vg appearance in serum; the doses required were 300 times that of E2, and the vitellogenic response was less when compared to the E2-induced response. In addition, a combination dose of methoxychlor with E2 decreased the magnitude of the response to E2. Other mammalian xenoestrogens,* o,p′-*DDT, chlordecone, lindane and ß-HCH, were not able to produce a vitellogenic response in this study. Copyright © 1996 Elsevier Science Ltd*

Xenoestrogens are compounds that have activity similar to the endogenous steroidal estrogens such as 17ß-estradiol (E2). E2 induces its biological effects by binding to a protein, the estrogen receptor (ER), and this steroid–receptor complex interacts with DNA to promote protein synthesis. Most xenoestrogens act by a similar mechanism but have less affinity for the ER, thus, less potency than E2. In mammals, several xenobiotics have been reported to have estrogenic activity. Activity is assayed by measuring stimulation of growth of the female reproductive tract (e.g. uterine/body weight ratios) in juvenile

rodents, competition for E2 binding sites in the uterus and promotion of proliferation in estrogen-responsive cell lines. Exposure to xenoestrogens has been reported in mammals to have both reproductive and developmental consequences purportedly by altering the hormonal milieu.

Fish are also adversely affected by environmental estrogens and can serve as animal models for studying estrogenic activity because of their estrogen-sensitive reproductive physiology. In the mature female teleost, the primary target tissue of E2 is the liver. Following E2 interaction with the ER, hepatocytes synthesize vitellogenin (Vg), a glycolipophosphoprotein which serves as the precursor for yolk proteins (Ng & Idler, 1983; Lazier & MacKay, 1993). In juvenile fish and normal males, exposure to estrogens can induce synthesis of the protein. Therefore, Vg is being examined as a marker of estrogenic activity in both *in vivo* and *in vitro* fish paradigms (Pelissero *et al.*, 1993; Purdom *et al.*, 1994).

The purpose of the present investigation was to determine if estrogenicity of select 'classical' mammalian xenoestrogens can be detected using an *in vivo* teleostean model. Ability of these compounds to alter the normal response to E2 was also examined since a hypothesis of the reproductive toxicity of xenoestrogens is that weakly binding compounds may be antagonists of endogenous estradiol.

Juvenile channel catfish (65–95 g) were anaesthetized with MS-222 and exposed to compounds by a single intraperitoneal (i.p.) injection of a 0.25% agar suspension on day 0. Each experiment included a negative control group, the highest volume of vehicle (10 ml/kg) and a positive control group (0.6 mg E2/kg; ED_{50} for Vg production). Preliminary exposures were used to examine each potentially estrogenic compound to determine the highest tolerated dose, as well as indication of estrogenic activity. Compounds tested included *o,p'*-DDT, methoxychlor, chlordecone, lindane, β-hexachlorocyclohexane (β-HCH) and *p*-nonylphenol (NP). The pesticides and isomers were obtained from Radian Corporation, and NP was a gift from Schenectady International (CAS RN 84852-15-3*). Treatment groups in these experiments had between three and four fish. If there was no Vg induction observed after exposure to the highest dose, these compounds were deemed void of estrogenic activity in the paradigm. The highest dose was limited by lethality or volume of injection. For compounds that appeared to have estrogenic activity in the preliminary phase, a follow-up experiment was conducted with larger sample sizes. Treatments included negative control, positive control, two dose groups of the xenobiotic and two combination groups in which the fish received the 0.6 mg E2/kg simultaneously with two levels of the xenobiotic. The synthetic estrogens ethinylestradiol, mestranol and diethylstilbestrol (DES), as well as the antiestrogen tamoxifen, were examined for estrogenic activity in similar experiments. On day 7, blood samples were taken from the caudal vein and fish were sexed by internal examination; liver weights were recorded. The hepatic–somatic index (HSI) was determined as the ratio of liver weight:body weight. Collected serum was stored at −60°C until assayed for Vg using an enzyme-linked immunosorbent assay utilizing monoclonal antibodies specific for channel catfish Vg (Goodwin *et al.*, 1992). Vg used in the assay was partially purified by precipitation with EDTA and $MgCl_2$ from the serum of E2-treated females.

Ethinylestradiol and mestranol treatment resulted in both higher Vg serum levels and greater hepatic–somatic indices (HSI) than E2 exposure (Table 1). The xenobiotic dose is expressed as the actual dose as well as the equimolar dose of E2. Therefore, at the same molar dose of E2, these synthetic steroidal estrogens had a higher potency for Vg induction and HSI increase. The higher potency of these compounds is consistent with the

TABLE 1
Estrogenic Activity of Xenobiotics

Treatment	*n*	Dose (mg/kg)	Estradiol equimolar dose (mg/kg)	Estrogenic parameters[a]			
				Vg(mg/ml)		HSI	
Agar (negative)	7	—	—	0.01	(0.01)	0.010	(0.002)
E2 (positive)	8	0.6	—	240.8	(120.0)	0.012	(0.002)
Ethinylestradiol	8	0.66	0.6	473.1[b]	(187.7)	0.014[b]	(0.003)
Mestranol	8	0.68	0.6	505.0[b]	(126.8)	0.015[b]	(0.002)
Ethinylestradiol + E2	7			459.5[b]	(104.5)	0.018[b]	(0.004)
Mestranol + E2	7			455.5[b]	(99.3)	0.019[b]	(0.002)
Agar	8	—	—	0.4	(0.2)	0.013	(0.002)
E2	8	0.6	—	323.6	(172.7)	0.014	(0.002)
DES	8	0.59	0.6	114.3[b]	(96.8)	0.012	(0.002)
DES + E2	8			634.7[b]	(231.5)	0.017[c]	(0.002)
Low tamoxifen + E2	8			267.9	(54.4)	0.015	(0.002)
High tamoxifen + E2	8			37.5[b]	(25.3)	0.013	(0.002)

[a]The values displayed are the means (standard deviation). [b]Values are significantly different from the E2 treatment ($P < 0.05$, ANOVA followed by Tukey's post-hoc test). [c]Values are significantly different from the agar treatment ($P < 0.05$, ANOVA followed by Tukey's post-hoc test).

reported higher binding affinity of ethinylestradiol for the ER, 158% compared to E2 (Wingard *et al.*, 1991). Mestranol is *o*-demethylated to ethinylestradiol to render it biologically active; this same metabolism is apparent in catfish.

The results for the non-steroidal agonist DES and antagonist tamoxifen are shown in Table 1. At the same molar dose as E2, DES increased Vg levels, but was not as potent as E2. DES was at least additive with E2, increasing Vg levels above the positive control dose as well as increasing the HSI above the negative control. In rodents, DES also has a high binding affinity for the mammalian ER, 141% (Wingard *et al.*, 1991). In a teleost, Smith & Thomas (1990) reported DES to be as effective in displacing radiolabeled E2 from binding sites in the spotted seatrout liver. Yet, in the present investigation, DES was not as effective as E2 in inducing the vitellogenic response. Pelissero *et al.* (1993) also reported that DES was less effective in stimulating isolated rainbow trout hepatocytes to produce Vg.

Tamoxifen, an antagonist at the ER, had no estrogenic activity at a dose more than 100 times the E2 dose (data not shown); however, when given in combination with E2, this dose significantly reduced the vitellogenic response by an order of magnitude (Table 1). The data in Table 1 validated the fish model of estrogenicity by giving evidence of a receptor-mediated mechanism and similar binding specificity as that in mammals.

Four of the compounds tested, chlordecone, lindane, β-HCH and *o,p'*-DDT, demonstrated no induction of vitellogenesis or changes in HSI compared to negative controls. The highest dose tested with lindane was 106.7 mg/kg and the highest chlordecone dose was 90 mg/kg. Doses higher than these resulted in lethality. These doses are equimolar to 100 and 50 mg E2/kg, respectively. The highest doses of β-HCH and *o,p'*-DDT tested were 320 and 390 mg/kg; both of these are equimolar to 300 mg E2/kg. While these doses resulted in no visible toxicity, higher doses of DDT and β-HCH were impractical. Low chronic administration of β-HCH has been reported to be estrogenic in medaka and

guppies on the basis of histological changes associated with induced vitellogenesis (Wester *et al.*, 1985; Wester & Canton, 1986). Denison *et al.* (1981) correlated the presence of a 'vitellogenin-like' protein identified by PAGE/Coomassie with DDT resistance in mosquitofish. The absence of biological activity of the DDT isomer investigated here is consistent with results of a competitive binding study which found *o,p'*-DDT did not bind to the ER in spotted seatrout liver (Thomas & Smith, 1993). The inactivity of chlordecone was inconsistent with its affinity for the seatrout ER (Thomas & Smith, 1993). However, in this present *in vivo* study, because high doses were not tolerated by fish, the threshold concentration of chlordecone at the ER may not have been reached. It may be the case that chlordecone requires metabolic activation which occurs in rats and seatrout but not channel catfish.

In Table 2, both the low and high doses of *p*-nonylphenol increased the levels of Vg as compared to negative controls. Individual fish varied in response, such that only the high dose was significantly different ($P < 0.05$). The range of individual responses in the low dose was 0.58–9.99 mg/ml and 0.11–14.71 mg/ml for the high dose. *p*-Nonylphenol coadministration with E2 did not alter the vitellogenic response. *p*-Nonylphenol and related compounds (octylphenol, carboxylic acid derivatives) are estrogenic in isolated rainbow trout hepatocytes; the potencies of Vg induction are 10,000–1,000,000 times less than E2 (Jobling & Sumpter, 1993). These compounds also possess affinity for rainbow trout ER *in vitro* albeit 1000 times less than E2 (White *et al.*, 1994). The high dose of *p*-nonylphenol in the present investigation was 500 times less than E2, and Vg levels were more than an order of magnitude less.

The vitellogenic effect of methoxychlor, shown in Table 2, is not significantly different from the negative control due to considerable variation in individual response. However, the range of serum Vg levels for the low dose was 0.27–9.99 mg/ml and for the high dose was 0.03–13.75 mg/ml. The high dose of methoxychlor resulted in a significant decrease of the response to E2. Three of the fish in this treatment group died in the first

TABLE 2
Estrogenic Activity of *p*-Nonylphenol and Methoxychlor

Treatment	*n*	Dose (mg/kg)	Estradiol equimolar dose (mg/kg)	Estrogenic parameters[a]			
				Vg(mg/ml)		HSI	
Agar	7	—	—	0.3	(0.4)	0.015	(0.002)
Low nonylphenol	7	79	100	3.6	(3.4)	0.015	(0.002)
High nonylphenol	6	237	300	9.5[c]	(5.7)	0.014	(0.002)
E2	7	0.6	—	355.5	(139.4)	0.017	(0.002)
Low nonylphenol + E2	8			368.4	(138.9)	0.017	(0.002)
High nonylphenol + E2	7			269.0	(133.1)	0.017	(0.003)
Agar	8	—	—	0.1	(0.1)	0.014	(0.002)
Low methoxychlor	8	127	100	2.5	(3.3)	0.014	(0.002)
High methoxychlor	8	380	300	3.3	(5.1)	0.011	(0.004)
E2	7	0.6	—	370.0	(98.6)	0.017	(0.003)
Low methoxychlor + E2	8			266.9	(81.1)	0.015	(0.002)
High methoxychlor + E2	5			125.5[b]	(141.7)	0.013	(0.003)

[a]The values displayed are the means (standard deviation). [b]Values are significantly different from the E2 treatment ($P < 0.05$, ANOVA followed by Tukey's post-hoc test). [c]Values are significantly different from the agar treatment ($P < 0.05$, ANOVA followed by Tukey's post-hoc test).

24 h post-injection and, therefore, this effect may be not be limited to specific estrogenic antagonism. The literature available on the potential estrogenic potency of methoxychlor in fish has been conducted *in vitro* by Thomas & Smith (1993) who reported methoxychlor to be devoid of binding affinity. This is consistent with the fact that in mammals methoxychlor is a proestrogen, requiring demethylation to a hydroxylated metabolite (Bulger *et al.*, 1985).

While the liver in fish is the target organ of E2, increases in HSI were only observed following treatment by the potent synthetic estrogens. In the dose-response curve used to establish the ED_{50} of E2 Vg induction, increases in the HSI were not observed until between 1 and 10 mg/kg whereas increased Vg was observed between 0.01 and 0.1 mg/kg. Therefore, the sensitivity of vitellogenesis makes it the preferred indicator of estrogenicity. Also, unlike uterine weight in mammals, changes in fish liver weight may be indicative of biochemical adaptations other than interaction with the ER, i.e. hypertrophy accompanying induction of metabolizing enzymes.

The actual values of serum Vg reported here are higher than previously reported in the literature and some values appear to exceed solubility. Inflation of values most likely has occurred due to the nature of the 'semi-purified' Vg used in the ELISA and as standards. While this 'semi-purified' Vg appeared free of other proteins when analyzed by SDS–PAGE/Coomassie, salts from the extraction may have increased the apparent weight when solutions were prepared. Therefore, all alterations in vitellogenesis have been compared only within-experiment to the positive and negative controls.

The *in vivo* vitellogenesis paradigm for ER activation in channel catfish appears to be consistent with mammalian literature when synthetic estrogens and antiestrogens are tested. The estrogenicity associated with *p*-nonylphenol and methoxychlor was demonstrated by the acute induction of vitellogenesis following i.p. injection. However, there is wide variation in the vitellogenic response in individual fish to both xenoestrogens and E2. These differences do not appear to be due to gender or size (within the established range), but sample sizes were not large enough to permit this kind of analysis. Another problem with the *in vivo* model was the toxicity associated with the pesticides. A low, chronic exposure to the pesticides which had no activity here, most notably the more toxic β-HCH and chlordecone, may result in a vitellogenic response in catfish. Compared to currently available literature, this study reveals species differences among fish in sensitivity to estrogenic activity, possibly due to binding affinities or metabolism.

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